

Calnexin and calreticulin bind to enzymically active tissue-type plasminogen activator during biosynthesis and are not required for folding to the native conformation

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The roles of the endoplasmic-reticulum lectins calnexin and calreticulin in the folding of tissue-type plasminogen activator (tPA) have been investigated using an *in vitro* translation system that reconstitutes these processes as they would occur in the intact cell. Using co-immunoprecipitation of newly synthesized tPA with antibodies to calnexin and calreticulin, it was demonstrated that the interaction of tPA with both lectins was dependent upon tPA glycosylation and glucosidase trimming. When tPA was synthesized in the presence of semi-permeabilized

cells under conditions preventing complex formation with calnexin and calreticulin, the translation product had a specific plasminogenolytic activity identical with that when synthesized under conditions permitting interactions with both lectins. Furthermore, complexes of tPA bound to calnexin and calreticulin were shown to be enzymically active. These results demonstrate that calnexin and calreticulin can form a stable interaction with correctly folded tPA; however, such interactions are not required for the synthesis of enzymically active tPA.

INTRODUCTION

Calnexin, a 64 kDa transmembrane protein, and calreticulin, a 45 kDa soluble luminal homologue of calnexin [1], are thought to be involved in protein folding, retention and quality control in the endoplasmic reticulum (ER). Calnexin was initially demonstrated to interact with assembling MHC class I molecules [2] in the ER, and has since been shown to interact with a diverse set of glycoproteins [3]. Several lines of evidence suggest calnexin is a component of the ER quality-control system. Dissociation of calnexin from proteins correlates well with maturation events. During the folding of MHC class I [4], influenza haemagglutinin (HA) [5–7] and transferrin [3], calnexin dissociation correlates with completion of disulphide-bond formation. During MHC class I assembly, calnexin dissociates on formation of heavy-chain- β_2 -microglobulin dimers [8,9]. Exit of MHC class I [2] and MHC class II [10] from the ER closely follows calnexin dissociation. Evidence that calnexin can prevent export of unassembled oligomeric complexes was provided by co-expression of calnexin with either MHC class I heavy chains, heavy-chain- β_2 -microglobulin dimers [11] or the T-cell receptor ϵ -subunit [12] in *Drosophila melanogaster* cells. The above studies have provided correlative evidence that calnexin and calreticulin are involved in subunit assembly of oligomeric proteins. Direct evidence for such a function for calnexin and calreticulin has been provided by the observations that both lectins increase the efficiency of folding and assembly and suppress degradation of HA synthesized in microsomes [13]. Similarly, calnexin has been shown to have similar effects on MHC class I expressed in *D. melanogaster* cells [14].

Calnexin [7,15] and calreticulin [16,17] have been shown to associate specifically with proteins bearing monoglucosylated oligosaccharides. Parodi and co-workers [18] have demonstrated

that deglycosylated glycoproteins are reglycosylated by UDP-glucose:glycoprotein glucosyltransferase. This glucosyltransferase had been shown to be specific for oligosaccharides on non-native proteins with no activity towards oligosaccharides on native proteins [19]. These observations have led Helenius and co-workers to propose that as long as a protein is non-native it is a target for reglycosylation and hence a ligand for calnexin and calreticulin, and thus retained in the ER [1,5,7,16]. Additionally, there has been a number of non-glycosylated proteins shown to co-precipitate with calnexin antibodies [12,20,21], and some glycoproteins have been shown to remain calnexin bound on enzymic deglycosylation [15]. These observations led to the notion that the lectin interaction is a prelude to a stronger protein-protein interaction [15]. This concept is now losing favour for a number of reasons. For example, glucosidase-deficient cells are devoid of substrates binding to calnexin [22]. A study with vesicular stomatitis virus G protein has demonstrated that the protein will co-immunoprecipitate with calnexin in glycosylation-dependent fashion; however, the non-glycosylated protein will only co-immunoprecipitate if aggregated [23]. Finally, two recent studies have shown that binding of ribonuclease to calnexin and calreticulin is independent of protein conformation and solely dependent upon whether the protein bears monoglucosylated oligosaccharides [24,25]. These studies have cast doubt on the functional relevance of non-glycosylated and deglycosylated proteins forming stable interactions with calnexin and calreticulin and suggest that an aggregation phenomenon is responsible. This has favoured the idea that calnexin and calreticulin function exclusively as lectins.

To extend these studies with a different model protein, we have used acquisition of enzymic activity, the definitive measure of protein folding, to investigate the specificities and roles of interactions of calnexin and calreticulin with glycoproteins. Using

Abbreviations used: BIP, Ig heavy-chain-binding protein; CHO, Chinese hamster ovary; dNM, deoxynojirimycin; ER, endoplasmic reticulum; HA, influenza haemagglutinin; IAA, iodoacetic acid; NLT, benzoyl-asparaginyl-leucinyl-threoninyl-*N*-methylamide; SP-cells, semi-permeabilized cells; tPA, tissue-type plasminogen activator.

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co-immunoprecipitation of tissue-type plasminogen activator (tPA) with antisera raised to calnexin and calreticulin and analysis of the precipitates by SDS/PAGE, it was demonstrated that tPA interacts with both lectins during its synthesis in a manner dependent upon glucose trimming of its oligosaccharides. To investigate whether the binding of calnexin and calreticulin was required for the correct folding of tPA, we studied the kinetics of folding of tPA synthesized in the presence or absence of deoxynojirimycin (dNM) in semi-permeabilized cells (SP-cells). No significant difference in the specific activity of tPA synthesized in the presence and the absence of dNM was observed. This demonstrated that the oligosaccharide-dependent interactions of calnexin and calreticulin with tPA were not required for the folding of this protein. When calnexin and calreticulin immunoprecipitates were analysed for tPA activity, they were found to be enzymically active. This indicated that calnexin and calreticulin could form stable oligosaccharide-dependent interactions with native tPA during biosynthesis in the endoplasmic reticulum.

EXPERIMENTAL

Materials

The plasmid pKC3T containing cDNA coding for wild-type tPA was a gift from Dr. Mary-Jane Gething (University of Melbourne, Melbourne, Australia). The CEM and CEM-NK[®] cells were gifts from Dr. Peter Cresswell (University of Yale, New Haven, CT, U.S.A.). Benzoyl-asparaginyl-leucinyl-threoninyl-N-methylamide (NLT) was a gift from Dr. Stephen High (University of Manchester, Manchester, U.K.). Rabbit reticulocyte lysate (Flexi Lysate[®]), amino acid mixture lacking methionine, RNasin and T7 RNA polymerase were purchased from Promega (Madison, WI, U.S.A.). EASYTAG[®] [³⁵S]methionine was purchased from New England Nuclear (Dreiech, Germany). dNM was purchased from Oxford Glycosystems (Oxford, U.K.). Protein A-Sepharose 4B was purchased from Zymed (San Francisco, CA, U.S.A.). Tissue-culture reagents and mouse anti-(human tPA) monoclonal antibody (clone L172D) was purchased from Gibco Life Technologies (Glasgow, Scotland, U.K.). Goat anti-(human tPA) polyclonal antibody was purchased from Alpha Laboratories (Eastleigh, Hants., U.K.). Rabbit anti-(human calreticulin) polyclonal antibody was purchased from Cambridge Biosciences (Cambridge, U.K.). The polyclonal anti-(dog calnexin) rabbit serum was raised against a synthetic peptide corresponding to the cytoplasmic tail of dog calnexin [26]. Dog pancreas microsomes were prepared using the method of Austen and co-workers [27]. ATP, CTP, GTP and UTP was purchased from Boehringer Mannheim (Lewis, East Sussex, U.K.). COA-SET tPA assay kit was purchased from Quadratch (London, U.K.). Ecocint A was purchased from National Diagnostics (Atlanta, GA, U.S.A.). All other reagents were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Preparation of SP-cells

The T-lymphoblastoid cell lines CEM and CEM-NK[®] [28] were maintained in Iscoves' modified Dulbecco's medium supplemented with 10% (v/v) fetal-calf serum. SP-cells were prepared by modification of the method described by Wilson and co-workers [29]. Sub-confluent CEM or CEM-NK[®] cells from 225 cm² flasks were pelleted at 720 g then resuspended and washed in 10 ml of ice-cold KHM (20 mM Hepes, pH 7.2, containing 110 mM potassium acetate and 2 mM magnesium acetate). The cells were pelleted and resuspended in 6 ml of KHM containing 40 µg/ml digitonin (diluted from a 40 mg/ml

stock in DMSO) then incubated on ice for 5 min. To terminate permeabilization, KHM (8 ml) was added, and the cells were pelleted then incubated in 50 mM Hepes, pH 7.2, containing 90 mM potassium acetate (10 ml), on ice for 10 min. The cells were pelleted and resuspended in KHM (100 µl). Endogenous mRNA was removed by adding calcium chloride to a concentration of 1 mM and staphylococcal nuclease to 10 µg/ml and incubating at room temperature for 12 min. The reaction was terminated by the addition of EGTA to a concentration of 4 mM. The cells were pelleted and resuspended in KHM (100 µl).

In vitro transcription and translation of tPA

Synthesis of wild-type tPA mRNA with T7 RNA polymerase has been described previously [30]. The mRNA transcript encoding wild-type tPA was translated and the ³⁵S-labelled products were translocated using the following translation reaction mixtures. When microsomes were used, each translation reaction consisted of 17.5 µl of Flexi Lysate, 0.5 µl of KCl (1 M), 0.5 µl of amino acids (2 mM) lacking methionine, 1 µl of nuclease-treated dog pancreas microsomes, 1 µl of EASYTAG[®] [³⁵S]methionine (10 µCi/µl), 3.5 µl of RNase-free water and 1 µl of tPA mRNA (1 mg/ml). When SP-cells were used, each translation reaction consisted of 17.5 µl of Flexi Lysate, 0.5 µl of KCl (1 M), 0.5 µl of amino acids (2 mM) lacking methionine, 4 µl of nuclease-treated SP-cells, 1 µl of EASYTAG[®] [³⁵S]methionine (10 µCi/µl), 0.5 µl of RNase-free water and 1 µl of tPA mRNA (1 mg/ml). Where indicated, NLT was added to a final concentration of 2 mM to inhibit core N-linked glycosylation. Where indicated, dNM was added to a final concentration of 1 mM to inhibit glucose trimming by glucosidase I and glucosidase II. The translation mixtures were incubated at 30 °C for 60 min unless otherwise indicated. When the NLT and dNM were included, the translation mixtures were pre-incubated at 30 °C for 10 min before the addition of tPA mRNA. All translations were terminated by alkylation, by adding iodoacetic acid to a final concentration of 20 mM followed by incubation on ice for 10 min; this also ensured that any remaining free thiols were blocked before further processing as described below.

Immunoprecipitation

For anti-tPA precipitation, alkylated translations were made up to a final volume of 1 ml with ice-cold Triton X-100 immunoprecipitation buffer [50 mM Tris/HCl, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 2 mM EDTA and 0.02% (w/v) sodium azide]. For anti-calnexin and anti-calreticulin precipitation, alkylated translations were made up to a final volume of 1 ml with ice-cold CHAPS immunoprecipitation buffer [50 mM Hepes, pH 7.4, containing 2% CHAPS, 200 mM NaCl and 0.02% (w/v) sodium azide]. The translations were pre-incubated with 50 µl of Protein A-Sepharose [10% (w/v) in PBS] for 60 min at 4 °C to preclear the samples of Protein A-binding components. Where SP-cells were used, insoluble cell debris was removed by centrifugation at 12000 g for 5 min after preclearing. Precleared samples were each incubated with 50 µl of Protein A-Sepharose and either 1 µg of monoclonal anti-tPA, 10 µl of polyclonal anti-calnexin serum or 8 µl of polyclonal anti-calreticulin serum for 15 h at 4 °C. The complexes were washed three times with the appropriate immunoprecipitation buffer, then prepared for analysis by SDS/PAGE as described below. Where indicated, calnexin and calreticulin immunoprecipitates were used directly in tPA assays described below. In this case the complexes were further washed with 1 ml of 50 mM Tris/HCl, pH 7.4, then resuspended in 80 µl of 50 mM Tris/HCl, pH 8.3.

SDS/PAGE

Immunoprecipitation pellets were resuspended in SDS/PAGE sample buffer [0.25 M Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 50 mM DTT, 20% (v/v) glycerol and 0.004% (w/v) Bromophenol Blue]. All samples were boiled for 5 min before electrophoresis. Cooled samples were loaded into the wells of a SDS/10%-polyacrylamide gel for electrophoresis. Gels were stained, fixed, dried under vacuum, and examined either by autoradiography using Kodak XAR-5 film or by a Fujix Bas2000 PhosphorImager.

Activity assays of tPA synthesized in SP-cells

Alkylated translations were centrifuged at 12000 *g* for 2 min, and the supernatant was removed. The cell pellet was washed with 1 ml of KHM, pelleted then resuspended in 20 μ l of TWS (50 mM Tris/HCl, pH 8.3) containing 0.01% (v/v) Tween-80. After incubation on ice for 1 h the cells were pelleted and the supernatant made up to 80 μ l with TWS. The tPA activities were determined using an indirect amidolytic assay previously described [31]. Conversion of the chromogenic substrate S-2251 into a coloured product by plasmin generated by the action of tPA on plasminogen was measured at 405 nm. The tPA activities were expressed in international units using human melanoma tPA (500000 units/mg) as a standard. To express the folding of tPA a specific activity measurement was required. This corrected for differences in translation efficiency between cell lines. The amount of tPA translated was estimated by scintillation counting of tPA immunoprecipitated using a non-conformational specific antibody from identical samples that were assayed. The specific activity of tPA, hence the degree of tPA folding in a cell extract, was expressed in arbitrary units of units/d.p.m. Where tPA was assayed in calnexin and calreticulin immunoprecipitates, activities were expressed as units of tPA immunoprecipitated per translation, where a translation reaction was 25 μ l (m-units/translation).

RESULTS

Interaction of tPA with calnexin and calreticulin requires N-linked glycosylation of tPA

Previous studies have demonstrated that the binding of calnexin [3] and calreticulin [32] to newly synthesized secretory proteins is dependent upon the proteins bearing N-linked oligosaccharides. We have demonstrated previously that enzymically active tPA can be synthesized in rabbit reticulocyte lysate supplemented with dog pancreatic microsomes optimized for glycosylation, folding and disulphide-bond formation [33]. Thus the initial stages in the biosynthesis and folding of tPA can be reconstituted in a cell-free system. The same system was used in this report to investigate the binding of tPA to calnexin and calreticulin.

To determine whether newly synthesized tPA bound calnexin and calreticulin in an oligosaccharide-dependent manner, tPA translations performed in the presence or absence of the tripeptide NLT (0.2 mM) were immunoprecipitated with antibodies to calnexin and calreticulin and separated by SDS/PAGE under reducing conditions (Figure 1). Binding of tPA to calnexin and calreticulin was indicated by the ability of these antibodies to co-immunoprecipitate tPA. When tPA translations were immunoprecipitated with antibodies to calnexin (Figure 1, lane 2) and calreticulin (Figure 1, lane 3), a band was observed with the same electrophoretic mobility as tPA immunoprecipitated with the mouse anti-tPA monoclonal antibody L172D (Figure 1, lane 1). This indicated that, under conditions where tPA became glyco-

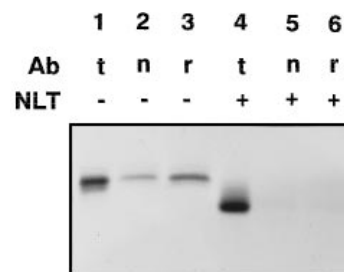


Figure 1 Association of tPA with calnexin and calreticulin is dependent upon N-linked glycosylation

Samples of rabbit reticulocyte lysate supplemented with dog pancreatic microsomes were incubated at 30 °C for 10 min in the absence (lanes 1, 2 and 3) or presence (lanes 4, 5 and 6) of 0.2 mM NLT. RNA coding for tPA was added and translated at 30 °C for 60 min. The translations were alkylated with 20 mM iodoacetic acid. The translation products were immunoprecipitated before electrophoresis with either L172D antibody (Ab) (t) (lanes 1 and 4), rabbit anti-calnexin polyclonal antibody (n) (lanes 2 and 5) or rabbit anti-calreticulin polyclonal antibody (r) (lanes 3 and 6). The immunoprecipitates were separated by SDS/PAGE and analysed by autoradiography.

lated, a proportion of the tPA molecules were stably associated with both calnexin and calreticulin. When tPA was translated in the presence of NLT and immunoprecipitated (Figure 1, lane 4), a single band corresponding to unglycosylated tPA was observed. When identical translations were immunoprecipitated with antibodies to calnexin and calreticulin, no detectable amounts of tPA were co-immunoprecipitated with calnexin (Figure 1, lane 5) or calreticulin (Figure 1, lane 6). To rule out the possibility that tPA associated non-specifically with the antisera or the Protein A-Sepharose, immunoprecipitations of tPA synthesized in the presence or absence of NLT were performed using an antibody raised against the α -subunit of prolyl 4-hydroxylase, a protein absent from dog microsomes [34]. No detectable amounts of tPA precipitated with this antibody in either case (results not shown). These results indicated that, under conditions where N-linked glycosylation of tPA was inhibited, tPA did not form a stable interaction with either calnexin or calreticulin. This was in direct agreement with the observation that tPA synthesized in intact Chinese hamster ovary (CHO) cells undergoes an oligosaccharide-dependent interaction with calnexin and calreticulin that can be prevented by tunicamycin treatment [35].

Interaction of tPA with calnexin and calreticulin requires trimming of tPA oligosaccharides

To determine whether glucose trimming was required for tPA to bind calnexin and calreticulin, translations using mRNA encoding wild-type tPA were performed in the presence of dNM. The translation products were immunoprecipitated as described above, and the precipitates were separated by SDS/PAGE under reducing conditions (Figure 2). When tPA was synthesized in the absence of dNM and immunoprecipitated, the translation product migrated as a doublet band (Figure 2, lane 1). When identical translations were immunoprecipitated with antibodies to calnexin (Figure 2, lane 3) and calreticulin (Figure 2, lane 5), single bands with similar electrophoretic mobility to type I tPA (Figure 2, lane 1) were observed corresponding to tPA complexed with calnexin and calreticulin. When tPA was immunoprecipitated from a translation performed in the presence of dNM, the translation product migrated as a doublet band (Figure 2, lane 2) with a slightly lower electrophoretic mobility relative to tPA synthesized in the absence of dNM (Figure 2, lane 1). This indicated that the

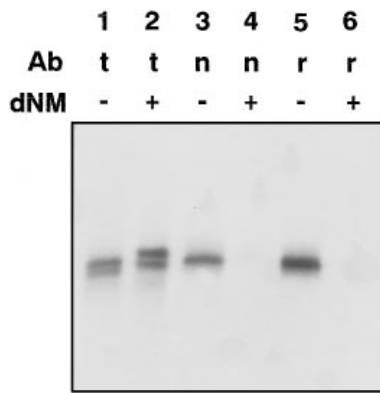


Figure 2 Glucosidase inhibitor dNM prevents the association of tPA with calnexin and calreticulin

Samples of rabbit reticulocyte lysate supplemented with dog pancreatic microsomes were incubated at 30 °C for 10 min in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 1 mM dNM. RNA coding for wild-type tPA was added and translated at 30 °C for 60 min. The translations were alkylated with 20 mM iodoacetic acid. The translations were immunoprecipitated with either L172D antibody (Ab) (t) (lanes 1 and 2), rabbit anti-calnexin polyclonal antibody (n) (lanes 3 and 4) or rabbit anti-calreticulin polyclonal antibody (r) (lanes 5 and 6). The immunoprecipitates were separated by SDS/PAGE and analysed by autoradiography.

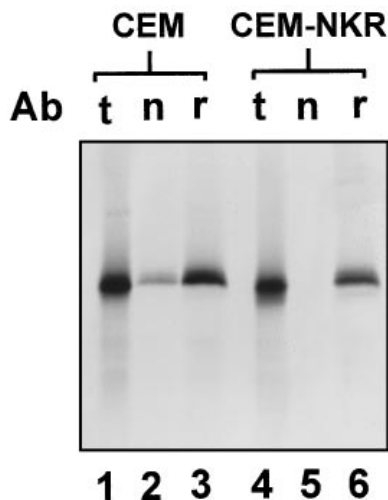


Figure 3 Association of tPA with calnexin and calreticulin in SP-cells

Samples of rabbit reticulocyte lysate supplemented with SP-CEM cells (lanes 1, 2 and 3) or SP-CEM-NK[®] cells (lanes 4, 5 and 6) were incubated at 30 °C for 60 min in the presence of wild-type tPA mRNA. The translations were alkylated with 20 mM iodoacetic acid. The translations were either immunoprecipitated with goat anti-tPA polyclonal antibody (Ab) (lanes 1 and 4), rabbit anti-calnexin polyclonal antibody (lanes 2 and 5) or rabbit anti-calreticulin polyclonal antibody (lanes 3 and 6). The immunoprecipitates were separated by SDS/PAGE and analysed by autoradiography.

trimming of the oligosaccharides of tPA had been inhibited by dNM. When identical translations were immunoprecipitated with antibodies to calnexin (Figure 2, lane 4) and calreticulin (Figure 2, lane 6), no detectable amounts of tPA co-immunoprecipitated with these antibodies. We concluded that under conditions where glucose trimming of tPA was inhibited, no stable interactions of tPA with calnexin and calreticulin were formed.

Folding of tPA in SP-cells

Previous work in our laboratory has shown that translocation, glycosylation and folding of tPA can be reconstituted using *in vitro* translation in the presence of SP-cells [29]. To determine whether tPA interacted with calnexin and calreticulin during synthesis, wild-type tPA mRNA was translated in the presence of SP-CEM cells. Immunoprecipitates from these translations using anti-tPA polyclonal antibody (Figure 3, lane 1), anti-calnexin polyclonal antibody (Figure 3, lane 2) and anti-calreticulin polyclonal antibody (Figure 3, lane 3) were analysed by SDS/PAGE (Figure 3). The calreticulin immunoprecipitate from CEM cells (Figure 3, lane 2) contained a band that co-migrated with tPA (Figure 3, lane 1). Similarly, the calreticulin immunoprecipitate from CEM cells (Figure 3, lane 3) contained a band that co-migrated with tPA (Figure 3, lanes 1). These results indicated that tPA synthesized in SP-CEM cells interacts with both calnexin and calreticulin. We have established that the oligosaccharide-dependent binding of tPA to calnexin and calreticulin could be abolished by dNM (Figure 2). It was reasoned that activity assays of tPA synthesized in the presence of glucosidase inhibitors could be used to assess any roles for oligosaccharide-dependent interaction of tPA with calnexin and calreticulin for the protein to fold to its mature plasminogenolytic conformation. This was achieved by translating mRNA encoding wild-type tPA in the presence of SP-CEM cells (Figure 4a) with or without dNM for various times up to 180 min. At each time point tPA synthesis and disulphide-bond formation were terminated by alkylation. The cells were isolated from the translation mixture and subjected to mild detergent lysis before determination of their tPA activity using a standard indirect chromogenic assay. The degree of folding of the tPA polypeptide at each time point was expressed as a specific plasminogenolytic activity measurement (Figure 4). Translations were also performed without wild-type tPA mRNA to control for possible interference of the tPA activity assay by endogenous proteases. When the assays were performed on translations without added tPA mRNA, no plasminogenolytic activities were detectable. When translations were performed in the presence of wild-type tPA mRNA, no difference in specific activity of the translation products were seen with or without dNM (Figure 4a). The experiment was repeated six times with similar results, and the data presented in Figure 4 are from one such experiment. Similar results were obtained when tPA was synthesized in the presence or absence of dNM using dog pancreatic microsomes instead of SP-cells (S. Allen and N. J. Bulleid, unpublished work). These results indicated that the rate of tPA folding when synthesized in the absence of oligosaccharide-dependent interactions with calnexin and calreticulin was identical to that of tPA that interacted with calnexin and calreticulin during synthesis. It was concluded that tPA can fold normally to its enzymically active conformation without oligosaccharide-dependent interactions with calnexin and calreticulin.

Folding of tPA in SP-cells that lack the calnexin gene product

Although the previous experiment demonstrated that inhibition of the oligosaccharide-dependent interaction of tPA with calnexin and calreticulin had no effect upon the ability of tPA to fold to its native enzymic conformation, it did not rule out the possibility that both lectins could act as molecular chaperones in the folding of tPA by interacting with the tPA polypeptide. To test whether tPA could fold in the absence of calnexin, we took advantage of the existence of the cell line CEM-NK[®], derived from the CEM cell line, that does not express the calnexin gene product [28,36,37]. To determine whether tPA interacted with calreticulin

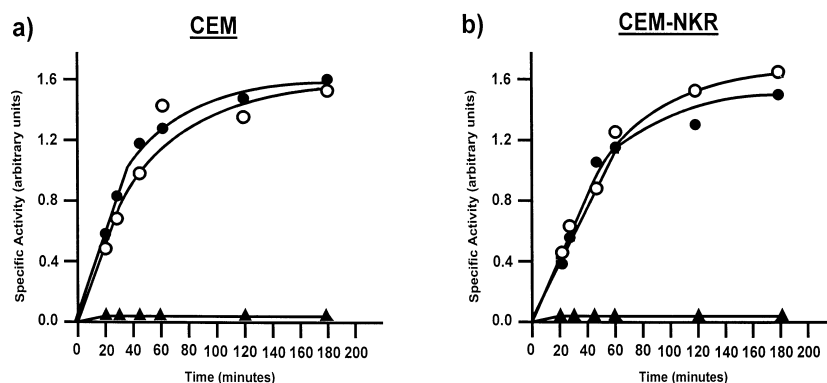


Figure 4 Folding of tPA synthesized in SP-cells in the presence or absence of dNM

Samples of rabbit reticulocyte lysate supplemented with SP-CEM cells (a) or SP-CEM-NK[®] cells (b) were incubated at 30 °C for 10 min in the absence (●) or presence (○) of 1 mM dNM. The samples were then incubated at 30 °C for 20, 30, 45, 60, 120 and 180 min in the absence (▲) or presence (● and ○) of mRNA encoding wild-type tPA. The translations were alkylated with 20 mM iodoacetic acid. The cells were isolated and lysed, and an indirect chromogenic assay [31] was used to determine the tPA activity (in units). The total amount of tPA for each time point of translation was estimated by immunoprecipitation and expressed in d.p.m. Folding of tPA was represented as an arbitrary specific plasminogenolytic activity measurement (units/d.p.m.) of the translation product as a function of translation time. The experiment was repeated six times with similar results. The data shown here are from one such experiment.

during synthesis in CEM-NK[®] cells, wild-type tPA mRNA was translated in the presence SP-CEM-NK[®] cells. Immunoprecipitates from these translations using anti-tPA polyclonal antibody (Figure 3, lane 4), anti-calnexin polyclonal antibody (Figure 3, lane 5) and anti-calreticulin polyclonal antibody (Figure 3, lane 6) were analysed by SDS/PAGE (Figure 3). The calnexin immunoprecipitate from CEM-NK[®] cells (Figure 3, lane 5) contained no detectable amounts of tPA, as expected from cells devoid of the calnexin gene product. The calreticulin immunoprecipitates from CEM-NK[®] cells (Figure 3, lane 6) contained a band that co-migrated with tPA (Figure 3, lane 4). These results indicated that tPA synthesized in SP-CEM-NK[®] cells interacts with calreticulin. To control for possible interference of the tPA activity assay by endogenous proteases, translations were performed without wild-type tPA mRNA. When the assays were performed on translations without added tPA mRNA, no plasminogenolytic activities were detectable (Figure 4b). When translations were performed in the presence of wild-type tPA mRNA, no differences in specific activity of the translation products were seen with or without dNM to prevent the oligosaccharide interactions of calreticulin with tPA (Figure 4b). Furthermore, no differences in the specific activity of the translation products were observed between CEM cells that expressed calnexin (Figure 4a) and CEM-NK[®] cells that lacked the calnexin gene product (Figure 4b). It was concluded that tPA could fold normally to its enzymically active conformation in the absence of any interaction with calnexin.

Complexes of tPA with calnexin and calreticulin contain plasminogenolytic activity

We have demonstrated above that tPA interacts with calnexin and calreticulin in an oligosaccharide-dependent manner and that disruption of these interactions has no effect on the ability of tPA to fold to an enzymically active conformation. Two separate studies have demonstrated recently that ribonuclease binds both calnexin and calreticulin in an oligosaccharide-dependent manner irrespective of whether the molecule is folded to a native conformation or not [24,25]. To test whether calnexin and calreticulin could bind to enzymically active, hence native, tPA, immunoprecipitates of tPA using antibodies to calnexin

Table 1 Activity of tPA in calnexin and calreticulin immunoprecipitates

Translation reactions supplemented with SP-CEM cells or SP-CEM-NKR cells were incubated at 30 °C for 60 min in the absence or presence of wild-type tPA mRNA. Products of translation were alkylated with 20 mM iodoacetic acid and immunoprecipitated with either calnexin- or calreticulin-specific antibodies. An indirect chromogenic assay [31] was used to determine the tPA activity associated with the immunoprecipitates and this is expressed as m-units/translation, where a translation reaction was 25 μ l. The results are mean activities from duplicate translations, except for those marked ^a, which are mean activities of immunoprecipitates from four translations. Figures in parentheses are from non-alkylated translations

Antibody	Cells	Activity (m-units/translation)	
		+ RNA	- RNA
Anti-calnexin	CEM	1.99 ^a (1.92)	0.09
	CEM-NKR	0.10 ^a (0.14)	0.12
Anti-calreticulin	CEM	0.71	0.04
	CEM-NKR	0.54	0.10

and calreticulin were assayed for plasminogenolytic activity due to associated tPA (Table 1). When translations were performed in the absence of tPA mRNA, no activity was detected in calnexin and calreticulin immunoprecipitates in both CEM and CEM-NK[®] cells. Also, no activity was detected in calnexin immunoprecipitates from tPA mRNA translated in the presence of CEM-NK[®] cells. These results ruled out the possibility of endogenous protease activity bound to calnexin and calreticulin precipitating with the respective antisera. However, when translations were performed in the presence of tPA mRNA, both calnexin and calreticulin immunoprecipitates from CEM cells and calreticulin immunoprecipitates from CEM-NK[®] cells exhibited plasminogenolytic activity due to associated tPA. We have also obtained similar results during the biosynthesis of tPA in intact cells in culture. When extracts prepared from intact CHO cells stably transfected with human tPA cDNA were immunoprecipitated with the polyclonal antibody raised against

calnexin, the tPA associated with calnexin was shown to be enzymically active (S. Allen and N. J. Bulleid, unpublished work). These results demonstrate that tPA when in its native and enzymically active conformation can bind to both calnexin and calreticulin in an oligosaccharide-dependent manner.

To determine whether the active tPA bound to calnexin was already active before immunoprecipitation, the activity of a calnexin immunoprecipitate from a translation that had been treated with the alkylating agent IAA before immunoprecipitation was compared with a calnexin immunoprecipitate from a translation that had not been alkylated (Table 1). When tPA mRNA was translated in the presence of SP-CEM-NK[®] cells, no plasminogenolytic activity was detected in calnexin immunoprecipitates, as calnexin was absent from these cells. However, when translations were performed in the presence of SP-CEM cells, the plasminogenolytic activities of the immunoprecipitates were similar even when the translations were alkylated with IAA before immunoprecipitation. This indicates that the tPA in the calnexin immunoprecipitate was folded and enzymically active before immunoprecipitation, as alkylation of unfolded tPA prevents further folding. Thus the plasminogenolytic nature of the lectin co-immunoprecipitates was not due to tPA folding during immunoprecipitation.

DISCUSSION

The precise role of calnexin and calreticulin during the folding and assembly of glycoproteins has been the subject of intense interest in recent years. A direct role for calnexin and calreticulin has been shown during the folding and assembly of HA and MHC class I molecules [13,14]. However, there are cases where preventing this binding to calnexin and calreticulin has little effect on protein secretion. There are members of a group of glycoproteins, which includes α -fetoprotein and transferrin, that are secreted efficiently in the presence of glucosidase inhibitors. Members of another group of glycoproteins, such as α_1 -acid glycoprotein, α_1 -antichymotrypsin, α_1 -antitrypsin, ceruloplasmin and α_2 -macroglobulin, are secreted, but at a reduced rate [5,38–40]. Also, human MHC class I transport and cell-surface expression are unaffected in a leukaemic cell line that does not express calnexin [36,37]. Clearly, for a number of secretory proteins, the roles of calnexin and calreticulin in these processes may not be essential.

This study addresses the role of these proteins in the folding of a soluble glycoprotein tPA. To date, all studies concerning the role of calnexin and calreticulin in glycoprotein synthesis have measured protein folding and assembly using conformational probes such as immunoprecipitation with conformation-specific antibodies, protease-protection assays and disulphide-bond formation using non-reducing SDS/PAGE. However, no study has measured enzymic activity of a glycoprotein, which is the definitive measure of native protein conformation, to assess the roles of calnexin and calreticulin in glycoprotein folding. We have shown previously that enzymically active tPA can be synthesized in a cell-free translation system supplemented with dog pancreatic microsomes [33]. To assess the effects of calnexin and calreticulin binding on the folding of tPA, we compared the enzymic activity of tPA synthesized under conditions where interaction with calnexin and calreticulin was allowed or prevented. The binding of tPA to calnexin and calreticulin was abolished when the polypeptide was synthesized in the presence of the glucosidase I and II inhibitor dNM. When tPA was synthesized in the presence of dNM, tPA was shown to fold to an enzymically active conformation as efficiently as tPA binding calnexin and calreticulin during synthesis. This result demon-

strates conclusively that oligosaccharide-dependent binding of calnexin and calreticulin to tPA is not required for this protein to fold to its native enzymically active conformation.

Consideration of the distance between pairs of cysteine residues involved in disulphide-bond formation in tPA compared with that between those residues in HA [41] that undergo incorrect disulphide-bond formation in the presence of glucosidase inhibitors may in part explain the varied dependency of folding upon calnexin and calreticulin. The HA monomer contains loop structures formed by Cys-14 with Cys-466 and Cys-52 with Cys-277 [41]. The N-terminal-most cysteine residues of pairs can reside within the lumen for prolonged periods until the partners emerge from the translocon. Calreticulin remains bound until a disulphide bond forms between Cys-52 and Cys-277. Throughout binding to calreticulin, HA is bound to calnexin. Calnexin dissociates on disulphide-bond formation between Cys-14 and Cys-466 [6,16,42,43]. Here, calnexin and calreticulin may serve to prevent aggregation, incorrect disulphide bonding and degradation. In contrast with HA, most of the disulphide bonds in tPA are between cysteine residues that are close in the primary sequence [44]. Although the pathway of disulphide-bond formation for tPA has not yet been determined, we have shown that disulphide-bond formation is near completion immediately after synthesis [45]. It is reasonable to assume that after translocation into the ER lumen unpaired cysteines do not have to wait long for their partners to emerge from the translocon. In most cases the C-terminal-most cysteine of a disulphide bond will be within the translocon before its partner has entered the ER lumen. This would reduce the propensity for incorrect disulphide-bond formation and facilitate efficient internalization of hydrophobic domains even in the absence of molecular chaperones. Previous studies have demonstrated that newly synthesized tPA in CHO cells undergoes weak transient interaction with the molecular chaperone Ig heavy-chain-binding protein (BiP) [46,47]. This interaction is increased by deletion of all three glycosylation sites by mutagenesis or inhibition of glycosylation by tunicamycin [46]. Furthermore, reduction of BiP expression increases the efficiency of tPA secretion [47]. The ability of tPA to fold in the absence of calnexin and calreticulin may be due to some degree of redundancy of chaperone function, in that interactions with chaperones such as BiP are sufficient to replace the role of lectin chaperones. This has also been suggested to explain the ability of MHC class I to fold, assemble and travel to the cell surface as normal in calnexin-deficient cells [36,37].

We demonstrated here that both calnexin and calreticulin interact with enzymically active tPA during biosynthesis in the ER. This observation shows that calnexin and calreticulin can bind to correctly folded tPA. These results extend those demonstrating the interaction of native and non-native ribonuclease with calnexin and calreticulin [24,25]. Zapun and co-workers [25] presented a model to describe the mode of binding of calnexin to its substrates. In this model calnexin acts exclusively as a lectin specific for monoglucosylated oligosaccharides irrespective of whether the substrate is folded or not. The association and dissociation with lectin is an equilibrium that is driven toward dissociation by deglycosylation of the protein by glucosidase II.

The demonstration here that calnexin and calreticulin bind tPA in a conformation-independent manner that does not influence acquisition of the native enzymically active conformation leaves an important question unanswered. What is the role of calnexin and calreticulin binding during biosynthesis in the ER? These lectins may serve to retain folding intermediates of tPA to facilitate efficient interactions with other proteins such as protein disulphide isomerase and BiP. Indeed the demonstration of interactions between resident ER chaperones and

redox enzymes [48–50] favours the idea that calnexin and calreticulin are part of a matrix providing structural framework for chaperones, redox enzymes and their substrates [1,50]. Further studies with a broader range of model glycoproteins are required so that the relationship between the structure of the model proteins and their dependence upon these lectin molecules for folding can be elucidated.

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